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Ascorbate (1g/day) does not help the phagocyte killing defect of X-linked chronic granulomatous disease

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SUMMARY

Three patients with X-linked chronic granulomatous disease of childhood (CGD) were treated with a single daily dose of 1 g of vitamin C over a period of 8 months. Prior to this clinical trial, isolated leucocytes from patients and normals were incubated with different concentrations of ascorbate and intracellular killing activity was investigated. Contrary to previous reports, there was no improvement of polymorphonuclear (PMN) intracellular killing activity after oral administration of ascorbate, nor could *in vitro* ascorbate correct defective, or enhance, killing activity of normal PMNs.

INTRODUCTION

Chronic granulomatous disease (CGD) is associated with increased susceptibility to pyogenic infections, and clinical manifestation of the disease starts in early childhood. There is impaired bactericidal activity of the leucocytes. The fundamental abnormality is the inability of the polymorphonuclear leucocytes (PMN) to undergo the burst of metabolic activity which normally accompanies phagocytosis (Holmes, Page & Good, 1967). The hexose monophosphate shunt (HMS) activity and generation of superoxide and H_2O_2 are defective in the phagocytes of CGD patients (Holmes *et al.*, 1967; Root *et al.*, 1975).

Other phagocytic parameters such as post-phagocytic degranulation, chemotaxis and random mobility are normal in these patients (Kaplan, Laxdal & Quie, 1968; Ward & Schlegel, 1969; Stossel, Root & Vaughan, 1972).

Ascorbic acid has been reported to increase HMS activity in CGD patients and has also been reported to be beneficial for patients suffering from Chediak–Higashi syndrome (Boxer *et al.*, 1976). There are reports of correction chemotaxis in three children said to have a combination of CGD and chemotaxis (Anderson, 1980). We therefore studied the *in vitro* effect of ascorbate on intracellular killing activity of three CGD patients and normal controls. Ascorbate was also administered to the patients orally at a dose of 1 g/day.

CASE HISTORIES

(1) R.C.

R.C. was born in 1967. He presented with cranial osteomyelitis at the age of 9 months when a diagnosis of chronic granulomatous disease (CGD) was made. Following spinal abscesses with aspergillus, an empyema and a subphrenic abscess, he was treated with a bone marrow transplant in January 1975. Only transient engraftment was achieved with 33% of neutrophils becoming nitro

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blue tetrazolium (NBT) reduction positive, during which seven sinuses healed up. However, within 3 months, immunological rejection of the HLA identical unrelated donor—previously mixed lymphocyte culture (MLC) compatible—occurred and infection recurred and persisted. Until the time of the study in 1981, R.C. had been admitted to hospital on numerous occasions for drainage of the neck, chest and subdiaphragmatic abscesses and for a spinal abscess which led to a paraplegia in 1976 which, however, recovered.

(2) D.M.

D.M. was born in 1968. A maternal uncle had died at the age of 2 years with chronic osteomyelitis, pneumonia and chronic hepatitis. The diagnosis of CGD was made in our patient at the age of 18 months following an episode of ascites and a scrotal infection. Following this, D.M. had several episodes of severe infection including pneumonias, a liver and a subphrenic abscess and had required numerous admissions to hospital.

(3) S.B.

S.B. was born in 1971 and diagnosed at birth as his elder brother was a known case of X-linked CGD and died of Gram negative septicaemia in 1972. For S.B. the first major problem was a submandibular abscess at 6 months. He had frequent upper respiratory tract infections associated otitis media, cervical lymphadenitis and a right upper lobe pneumonia. After two near fatal septicaemic episodes, an unrelated HLA identical MLC compatible donor was found, and S.B. received a bone marrow transplant (The Westminster Bone Marrow Transplant Team, 1977). This took to the extent of 12% of the peripheral blood neutrophils becoming both female (donor) and NBT positive and he remained virtually free of infection for nearly 7 years. During this period his graft slowly disappeared with never any evidence of immunological rejection.

From 1980, S.B. began to suffer recurrent infections typical of a relapse of his CGD, his cells were 100% male and NBT negative. An abscess of the lung was drained and shown to be due to *Streptotocci milleri.* Further episodes of septicaemia were documented.

At the time of the presentation all three boys were considered to have X-linked CGD and were suffering the recurrent infections required of the syndrome.

METHODS

Clinical trial. Patients were given a single dose of 1 gram oral vitamin C per day for 8 months and blood samples were taken every 2 months for assessment of intracellular killing of phagocytes.

Laboratory investigations

(a) Candida killing assay was performed by slight modification of the method described by Lehrer & Cline (1969) for killing of *Candida albicans* by phagocytes.

(1) Briefly a leucocyte rich fraction was prepared from peripheral blood by dextran sedimentation and the concentration of polymorphonuclear (PMN) leucocytes was adjusted to 1×10^7/ml after washing three times with TC199 medium $2{\cdot}5 \times 10^6$ PMN were mixed with an equal number of *C. albicans* in 25% plasma and were incubated with constant inversion agitation at 37°C for 1 hr. Sodium deoxycholate was then added to lyse the PMN and the percentage of dead Candida was assessed by a dye exclusion technique. In additional experiments, normal PMNs and those from the patients were incubated at 37°C for 15 and 30 min with concentrations of ascorbate ranging from 5 to 20 mM per culture and then washed three times with medium. After resuspension these leucocytes were also used for Candida killing assays.

(2) Nitro blue tetrazolium (NBT) test was carried out according to the method described by Hitzig (1973). A mixture of 1×10^7 PMN with 25% plasma and 1×10^7 killed Candida was incubated with 0·5% NBT for 30 min at 37°C after which time the percentage of phagocytic cells bearing formazan blue granules in their cytoplasm was calculated. A normal control was used throughout the experiments. Phagocytes treated with ascorbate as above were also used in this test.

RESULTS

In vitro Candida killing (Fig. 1) shows results of Candida killing tests using PMN which has been pre-incubated with or without ascorbate and indicates that ascorbate at the concentrations and incubation times used did not correct the defect in patients' PMNs or enhance killing activity of normal cells.

Results of Candida killing after oral administration of 1 g daily ascorbate are shown in Fig. 2, again indicating no improvement of Candida killing and NBT test.

The effects of pre-incubation of PMN with ascorbate on the NBT test was evaluated. Again, no improvement was observed for patients or controls.

DISCUSSION

The observed intracellular killing defect of granulocytes and the impaired NBT test in our patients suffering from recurrent infections since early childhood is typical of CGD.

There are reports of improvement of chemotaxis and intracellular killing activity of PMNs from

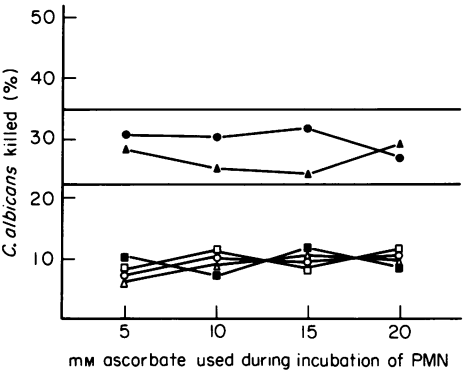

Fig. 1. The effect of different concentrations of ascorbate and incubation times on the candidicidal activity of polymorphonuclear leucocytes from patients R.C. and D.M. (horizontal lines enclose limits of normal range). ● = normal PMN, 15 min incubation; ▲ = normal PMN, 30 min incubation; ■ = R.C. PMN, 15 min incubation; □ = R.C. PMN, 30 min incubation; ○ = D.M. PMN, 15 min incubation; △ = D.M. PMN, 30 min incubation.

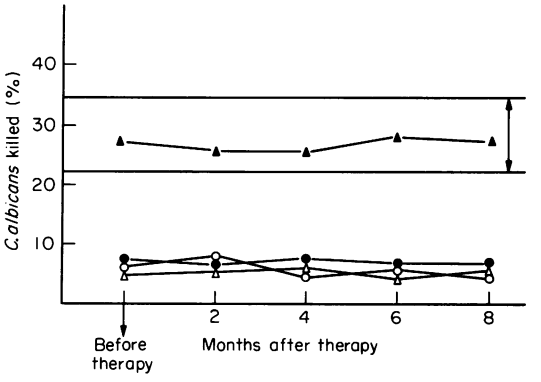

Fig. 2. The effect of daily oral ascorbate on the candidicidal activity of polymorphonuclear leucocytes from three CGD patients during 8 months of therapy (arrows represent limits of normal range). ▲ = normal; ● = R.C.; ○ = D.M.; △ = S.B.

patients with Chediak–Higashi syndrome (Boxer *et al.*, 1976) and CGD (Anderson, 1980) after treatment with ascorbate both *in vivo* and *in vitro*.

Incubation of granulocytes with ascorbate *in vitro* in our experiments did not enhance intracellular killing of Candida, nor did it produce any improvement in the NBT test, whether the granulocytes were of patient or normal origin.

Attempts to correct the intracellular killing defect in our CGD patients were not successful, despite continuous administration of a large dose of ascorbate for 8 months, as evidenced by the persistence of impaired Candida killing and NBT tests throughout the study.

Anderson's (1980) patients were suffering from a combination of CGD and chemotaxis defects and both *in vitro* and *in vivo* treatment with ascorbate improved laboratory and clinical findings in these patients. In other experiments performed in 1976 by Boxer *et al.* in patients with Chediak–Higashi syndrome *in vitro* and *in vivo* correction of the intracellular killing defect and cAMP and cGMP activity was also demonstrated.

It is possible that the discrepancy between our results and these previously reported findings may be due to our selection of patients with purely classical CGD; it seems that Anderson's patients had a combination of HMP shunt and chemotaxis defects which may be a different entity compared with classical CGD. This seems the more likely in that a sister was affected. Additionally, those patients may have defects in other enzyme systems within the HMP shunt which are correctable by ascorbate. It emphasises that the NBT test alone (negative in all three of Anderson's patients) is not an absolute diagnostic criterion for classical CGD.

We have not studied the effect of ascorbate therapy in the Chediak–Higashi syndrome, however, our experience demonstrates that ascorbate therapy cannot remedy the recurrent infections or correct the defective intracellular killing in our X-linked CGD patients and in the two true mothers the Lyon effect was clearly seen, only 50% of PMN being NBT positive.

REFERENCES

ANDERSON, R. (1980) Assessment of oral ascorbate in three children with chronic granulomatous disease and defective neutrophil mobility over a 2 year period. *Clin. exp. Immunol.* **43,** 180.

BOXER, L.A., WATANABE, A.M., RISTER, M., BESH, H.R., ALLEN, J. & BEAHNER, R.L. (1976) Correction of leukocyte function in Chediak–Higashi syndrome by ascorbate. *N. Engl. J. Med.* **295,** 1041.

HITZIG, W.H. (1973) Normal and defective phagocytosis—theory, clinical aspects and laboratory diagnosis. *Triangle*, **12,** 57.

HOLMES, B., PAGE, A.R. & GOOD, R.A. (1967) Studies of the metabolic activity of leukocytes from patients with a genetic abnormality of phagocyte function. *J. clin. Invest.* **46,** 1422.

KAPLAN, E.L., LAXDAL, T. & QUIE, P.G. (1968) Studies of polymorphonuclear leucocytes from patients with chronic granulomatous disease of childhood: bactericidal capacity for streptococci. *Pediatrics*, **41,** 591.

LEHRER, R.I. & CLINE, M.J. (1969) Interaction of *Candida albicans* with human leucocytes and serum. *J. Bact.* **98,** 996.

ROOT, R.K., METCALF, J., OSHINO, H. & CHANCE, B. (1975) H_2O_2 release from human granulocytes during phagocytosis. I. Documentation, quantitation and some regulating factors. *J. clin. Invest.* **55,** 945.

STOSSEL, T.P., ROOT, R.K. & VAUGHAN, M. (1972) Phagocytosis in chronic granulomatous disease and the Chediak–Higashi syndrome. *N. Engl. J. Med.* **286,** 120.

WARD, P.A. & SCHLEGEL, R. (1969) Impaired leucotactic responsiveness in a child with recurrent infections. *Lancet*, **ii,** 344.

THE WESTMINSTER HOSPITAL BONE MARROW TRANSPLANT TEAM (FOROOZANFAR, N., HOBBS, J.R., HUGH-JONES, K., HUMBLE, J.G., JAMES, D.C.O., SELWYN, S., WATSON, J.G. & YAMAMURA, M.) (1977) Bone Marrow Transplant from an unrelated donor for chronic granulomatous disease. *Lancet*, **i,** 210.